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Short Communication

Long-term Senolytic Treatment Prevents Endothelial Dysfunction in Arterial Aging

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ABSTRACT: Endothelial senescence is a critical contributor of arterial dysfunction and age-related cardiovascular diseases. This study demonstrates that long-term senolytic treatment with dasatinib plus quercetin (D+Q; 5 mg/kg + 50 mg/kg biweekly for 8 months) in mice significantly attenuates vascular endothelial senescence. D+Q lowered senescence markers (p21 protein and SA-β-gal positivity) in aged mesenteric arteries and human umbilical vein endothelial cells (HUVECs), while maintaining endothelial integrity. Transcriptomic analysis indicated activation of the relaxin signaling pathway and upregulation of nitric oxide synthase isoforms. Mechanistically, D+Q reversed age-related eNOS uncoupling by promoting dimerization, increased nitric oxide bioavailability, and reduced mitochondrial dysfunction, evidenced by restored mitochondrial ultrastructure, decreased mitochondrial mass, and lowered reactive oxygen species (ROS) production. Consequently, D+Q restored endothelium-dependent vasodilation and enhanced blood flow in aged mesenteric arteries following acetylcholine stimulation. These findings demonstrate that clearance of senescent endothelial cells via senolytic therapy mitigates arterial aging by restoring mitochondrial homeostasis and eNOS function, highlighting its therapeutic potential for age-related vascular dysfunction.

Keywords: Senolytics, Endothelial senescence, Arterial aging, Mitochondrial dysfunction

INTRODUCTION

Endothelial senescence plays a crucial role in the progression of arterial dysfunction and hastens the onset of various age-related cardiovascular diseases (CVDs). This phenomenon has been widely observed in multiple vascular organs, including the brain and the kidney, where it disrupts physiological functions and contributes to the development of conditions like hypertension, atherosclerosis, vascular dementia, myocardial infarction, pulmonary hypertension, and renal failure [1]. Hallmark features of endothelial senescence render individuals more susceptible to CVDs. These include a diminished ability for cell replication, reduced nitric oxide production, and increased inflammation, all of which contribute to the pathological alterations culminating in endothelial dysfunction [1]. Senescent cells exhibit a resistance to apoptosis, driven by the upregulation of specific proteins within the senescence cell anti-apoptotic pathways (SCAPs) [2]. Research has shown that the clearance of senescent cells, using transgenic or pharmaceutical interventions, can effectively delay the aging process, address age-related ailments, and prolong an individual's healthspan [3].

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Senolytics, a class of pharmaceutical compounds that target senescent cells, have emerged as a promising approach to eliminating these cells by inducing their apoptosis through pathways such as BCL-2 family members, p53, p38 and MAPK [4]. Among the first senolytic compounds discovered in 2015 are dasatinib (D) and quercetin (Q), each possessing unique activity profiles that together enhance their effectiveness in targeting senescent cells [2]. The therapeutic potential of combining dasatinib and quercetin (D+Q) has been demonstrated in various age-related conditions, including VSMCs dysfunction, skin rejuvenation, intestinal senescence, cognitive function, physical performance, and longevity [5, 6]. In this study, we investigate the impact of the senolytic combination of dasatinib plus quercetin (D+Q) treatment on vascular endothelial cells during aging [5].

MATERIALS AND METHODS

Mice

Male C57BL/6J mice were allocated into young (12–14 weeks), aged vehicle-treated (20 months), and aged senolytic-treated (D+Q, 20 months) groups. Mice were housed in individually ventilated cages under a 12-hour light/dark cycle with free access to food and water. All procedures complied with institutional guidelines and were approved by the local animal care committee of affiliated Nanshan Hospital of Shenzhen University.

Senolytics Treatment

Aged mice received dasatinib (5 mg/kg; Selleck, S1021) plus quercetin (50 mg/kg; Sigma, Q4951) dissolved in 10% PEG-400, administered orally twice weekly for eight months, as previously described [5]. Controls received vehicle. Body weight and general health were monitored throughout the study (Supplementary Fig. 1).

Histological and Cellular Senescence Assays

Senescence-associated β-galactosidase (SA-β-Gal) staining was performed in human umbilical vein endothelial cells (HUVECs) following the manufacturer's instructions (MedChemExpress, HY-K1089). Blue-stained cells were quantified under light microscopy.

Protein Expression Analysis

Western blotting was carried out on mesenteric arteries homogenized in RIPA buffer. Primary antibodies against eNOS (Abcam, #ab300071), p21 (Beyotime, #AP021), and α-tubulin (Abcam, #ab52866) were used. Bands were visualized using ECL reagent (Thermo Fisher) and quantified with ImageJ software.

Immunofluorescence

Mesenteric artery sections (8 μm) were fixed, permeabilized, and incubated with antibodies to p21 and CD31 (Abcam, #ab222783), followed by Alexa Fluor–conjugated secondary antibodies and DAPI counterstaining [5]. Confocal imaging was performed using an Olympus FluoView FV2000 microscope.

Mitochondrial and ROS Analyses

Transmission electron microscopy was used to assess mitochondrial morphology. JC-1 staining evaluated mitochondrial membrane potential. Intracellular and mitochondrial ROS were detected using DHE and MitoSOX probes (MedChemExpress) [7].

Nitric Oxide and Biochemical Assays

Nitric oxide levels were visualized in cryosections using DAF-FM DA fluorescence (Beyotime, #S0019S). BH4 concentrations were measured by competitive ELISA (Ruixin Biotech, JRXW2017586) [8].

RNA Sequencing

RNA was extracted from mesenteric arteries (n = 4 per group) using the Qiagen miRNeasy Mini Kit and sequenced by Novogene. Reads were aligned to the mouse genome (GRCm39) via STAR, and differential expression was analyzed using DESeq2 (FDR < 0.05).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 10.5. Normality was assessed with the Shapiro–Wilk test. Student's t-test, one-way ANOVA, or Kruskal–Wallis tests were applied as appropriate, with $p < 0.05$ considered significant.

RESULTS

Senolytic treatment attenuates vascular endothelial senescence

In this study, D+Q treatment was administered to 12-month-old mice over a period of 8 months, with doses given every two weeks at 5 mg/kg +50 mg/kg per mice (Fig. 1A). Senolytics have been proposed as a means of inducing apoptosis in senescent cells by inhibiting p21, p16INK4A, BCL-xL, PI3KCD, PAI1, and PAI2 [9].

Here, we examined the expression of p21 in mesenteric arteries treated with either vehicle or D+Q in order to assess the effects of D+Q on the aging process. In accordance with our previous data [5], an upregulation of p21 protein expression in the mesenteric artery was observed in aged mice. Conversely, a reduction in expression was noted following administration of D+Q in 20-month-old mice ($P < 0.01$) (Fig. 1B-E). Furthermore, we conducted immunofluorescence analysis of CD31 to specifically label ECs in the mesenteric arteries. Our fluorescent immunohistochemistry results supported the observation of p21 expression changes in the mesenteric ECs following D+Q treatment in aged mice ($P < 0.001$) (Supplementary Fig. 2).

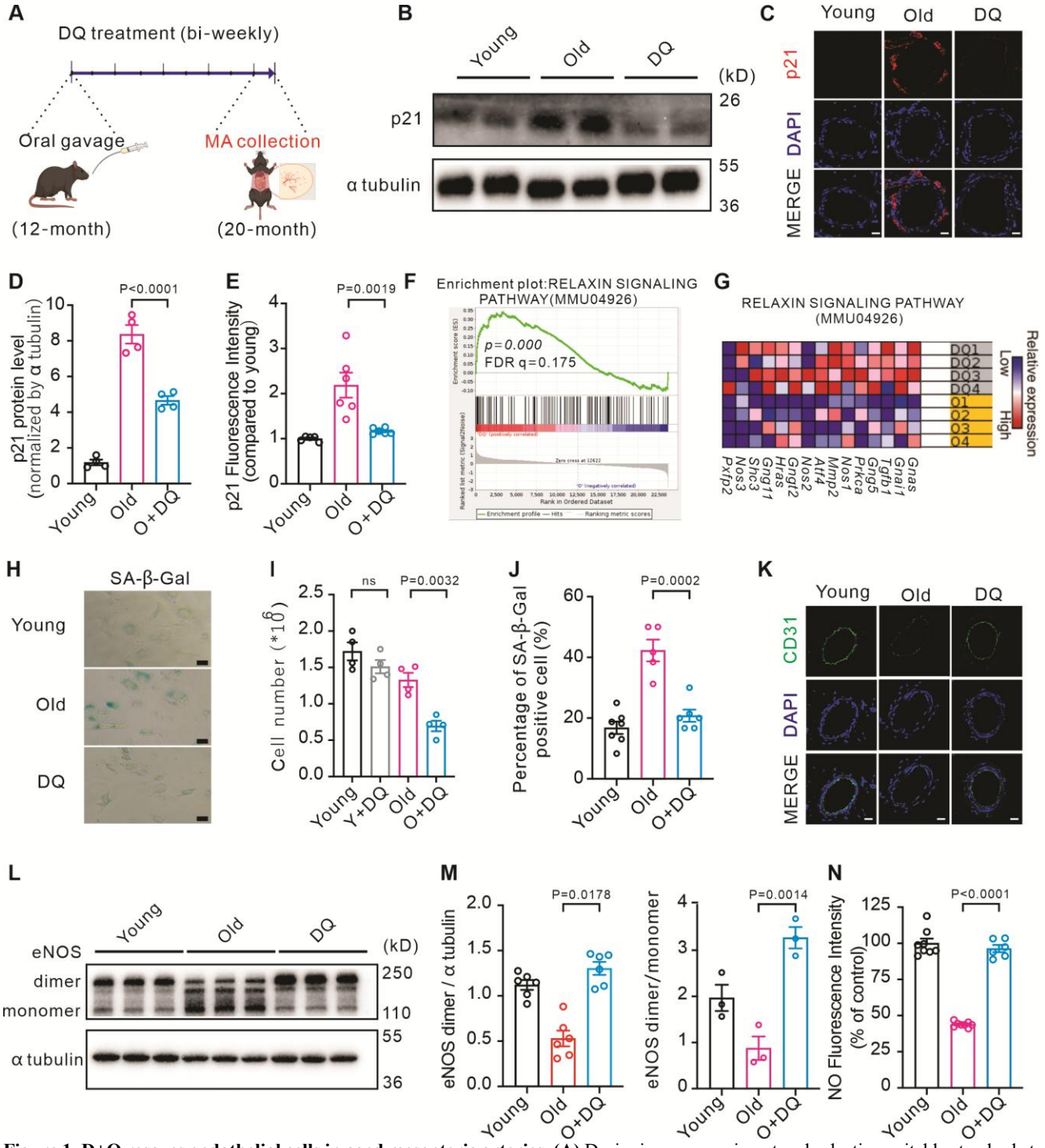

Figure 1. D+Q rescues endothelial cells in aged mesenteric arteries. (A) Designing an experiment and selecting suitable standards to evaluate the impact of D+Q on the vascular of aging mice. experimental design of D+Q treatment in aged mice. **(B-E)** Western blot

analysis (B) and fluorescent immunohistochemistry analysis (C) of p21 proteins in mesenteric arteries Aof aged versus D+Q mice. Scale bar, 20 mm. Summary of the results (D-E) (n = 4 for western blot analysis; n = 6 for Immunofluorescence analysis). **(F-G)** Shown are gene set enrichment analyses of positive regulation of relaxin signaling pathway (F) and heat maps for the top 15 up-regulated genes for RELAXIN SIGNALING PATHWAY (G) in D+Q compared with vehicle group. FDR, false discovery rate. **(H-J)** Representative images of senescence-associated β-gal (SA-β-Gal) staining in HUVECs treated with (+) or without (−) D+Q (0.2 μM+20 μM) (H). Scale bar: 50 μm. **(I-J)** Summary of the results (n = 4 for cell number counting analysis; n = 4 for SA-β-Gal staining analysis). **(K)** Representative images of CD31 staining in sections of mesenteric arteries (anti-CD31, green; DAPI, blue). Scale bars, 20 μm. **(L-M)** Western blot analysis of eNOS proteins in mesenteric arteries of aged versus D+Q mice (L) and quantification of western blot results (M) (n = 3 mice in each group). **(N)** Nitric oxide levels in mesenteric artery by using DAF-2DA fluorescence staining (young n = 8 arteries from 4 mice; old n = 8 arteries from 4mice; DQ n = 6 arteries from 4 mice). All quantitative data are presented as mean ± SEM. The normality of all quantitative data was verified using the Shapiro-Wilk test. Statistical significance was determined using Kruskal-Wallis followed by Dunn tests for panels D, E, I, J, M, and N. The number of biological replicates (n) is indicated in each bar graph. $P < 0.05$ was considered statistically significant.

Subsequent analysis was conducted on RNA sequencing data obtained from the mesenteric artery of aged mice treated with either vehicle or D+Q. Through Gene Set Enrichment Analysis (GSEA), we identified a multitude of signaling molecules and genes associated with the response to D+Q treatment, specifically highlighting the involvement of the RELAXIN SIGNALING PATHWAY (MMU04926) within the artery (Fig. 1F) (Supplementary Table 1). The peptide hormone relaxin mediates many biological actions including vasodilatory, anti-inflammatory, anti-apoptotic, and upregulation of vascular endothelial growth factor, which is partially linked to artery activity [10]. Moreover, the expression of nitric oxide synthase isoforms in the relaxin signaling pathway was markedly elevated in D+Q-treated mesenteric artery in this study (Fig. 1G). Thus, we hypothesize that D+Q exerts an impact on senescent ECs in aged mice.

Senescent ECs are proposed to contribute to vascular ageing and age-related vascular diseases. Clearance of senescent cells through senolytic agents has been demonstrated to alleviate vasomotor dysfunction in naturally aging mice [2]. However, the potential role of endothelium-derived relaxing factors in this process remains to be elucidated. To investigate the impact of senolytic therapy on ECs, we assessed the senescence-associated beta-galactosidase (SA-β-gal) levels in human umbilical vein endothelial cells (HUVECs), revealing 40% SA-β-gal-positive cells in aged (14^{th} passage, P14) HUVECs compared to 15% in young (4^{th} passage, P4) HUVECs ($P < 0.01$) (Fig. 1 H-J). The selective clearance of harmful senescent cells has the potential to reduce the negative effects of ageing microenvironment and improve tissue function in both aging and age-related diseases. Importantly, D+Q induces apoptosis strictly in senescent cells, rather than non-senescent controls observed in vitro. In alignment with prior investigations [11], our findings demonstrate that administration of D+Q led to a decrease in the proportion of SA-β-gal-positive cells in aged HUVECs, while exhibiting no apparent impact on younger cells (Fig. 1 H-J). Furthermore, the GSEA revealed a significant activation of gene sets related to the positive regulation of the apoptotic signaling pathway in aged mice treated with D+Q. While, gene sets associated with endothelial cell proliferation were not significantly altered by the treatment (Supplementary Table 2). This specific activation of apoptosis, in the absence of a broad impact on proliferative pathways, provides genomic evidence that D+Q selectively induces death in senescent cells of the endothelium. Moreover, our study revealed a higher incidence of endothelial damage in the mesenteric artery of aged mice as opposed to young, manifested by a decrease in intact endothelial cells (also see [12]). While this impairment was alleviated in mice that received treatment with D+Q (Fig. 1K). Collectively, these findings suggest that D+Q possesses senolytic properties by targeting senescent ECs, which potentially contribute to the maintenance of vascular function in aging arteries.

Endothelial nitric oxide synthase (eNOS) is predominantly found in vascular ECs and responsible for producing nitric oxide, thus playing a crucial role in maintaining vascular homeostasis [13]. Previous studies have shown that eNOS dimerization and the uncoupled state, rather than phosphorylation, are essential factors in the modulation of vascular dysfunction associated with aging [14]. We here investigated the expression of eNOS in mesenteric artery. Our results indicate that the age-related eNOS uncoupled state was effectively reversed following D+Q treatment ($P < 0.05$), leading to the promotion of eNOS dimerization (Fig. 1L-M). We next confirmed these results by measuring nitric oxide levels in mesenteric arteries. Consistent with previous findings [15], a decrease in nitric oxide levels was observed in the old group compared to young. Conversely, D+Q treatment promoted nitric oxide levels in aged mice ($P < 0.0001$) (Fig. 1N).

Senolytic treatment reduces mitochondrial ROS

To investigate the mechanisms underlying improved EC function, we conducted a comprehensive analysis utilizing reactome pathway analysis, which unveiled notable enrichment of genes involved in BIOLOGICAL OXIDATIONS networks (Fig. 2A). Moreover, GSEA

identified Positive Regulation Of Mitochondrion Organization (GO: 0010822) in response to D+Q treatment in aged arteries (Fig. 2B). Biological oxidation is well known to be an important contributor in age-associated EC and arterial dysfunction. Reactive oxygen species (ROS) are commonly understood as the byproducts of cellular respiration, particularly emanating from dysfunctional mitochondria [16]. Recent research has further elucidated the chemical reactivity and harmful effects of mitochondrial ROS, supporting the notion that dysfunctional mitochondria contribute to the accumulation of ROS during disease progression [17]. In this study, we first examined the mitochondrial ultrastructure in mesenteric ECs. Our findings demonstrated a reduction in mitochondrial mass in ECs treated with D+Q compared to vehicle group (Fig. 2C-D).

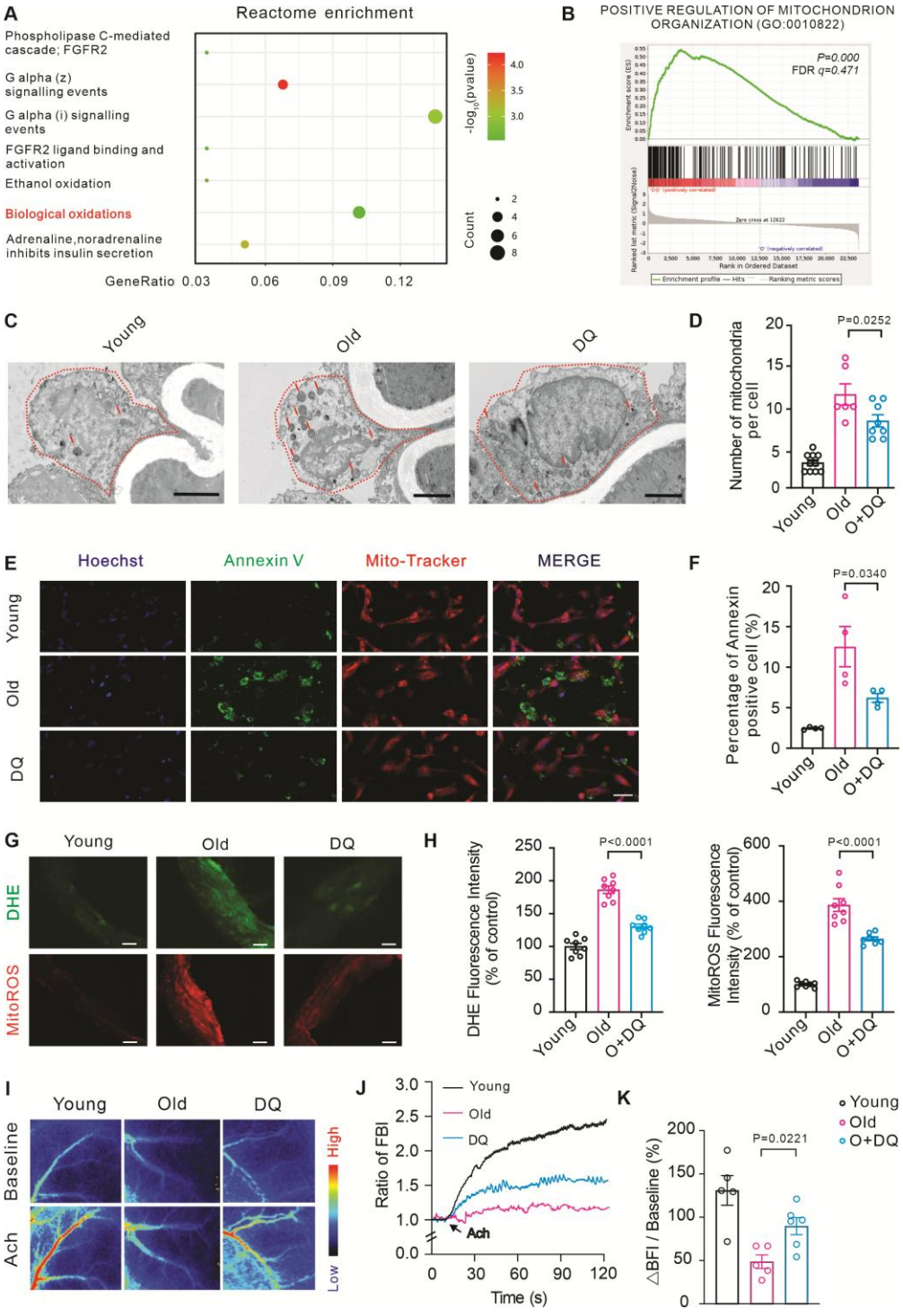

Figure 2. D+Q treatment attenuates mitochondrial dysfunction in aged vascular endothelial cells. **(A-B)** shown are reactome pathway analysis (A) and gene set enrichment analyses (B) of POSITIVE REGULATION OF MITOCHONDRION ORGANIZATION in D+Q compared with vehicle group. **(C-D)** electron microscopy image of mesenteric ECs from vehicle- and D+Q- treated mice (C), and summary of the results (D). Scale bar, 2.0 μm, (Young： n = 11 cells from 4 mice; Old: n = 6 cells from 4 mice; D+Q: n = 8 cells from 4 mice). **(E-F)** representative images of HUVECs stained with Annexin V, a marker of phosphatidylserine exposure during apoptosis (Annexin V, green; Mito-tracker, red; Hoechst, blue) (E) and quantitative analysis of the percentage of apoptotic cells (F). Scale bar,10 μm (n = 4). **(G-H)** representative fluorescent images of DHE staining and MitoSOX staining of the mesenteric aortas (G) and quantification of fluorescent results (H). Scale bar, 5 mm. **(I-K)** D+Q improves in vivo vasodilation in aged arteries. (I), representative pseudo-colored blood flow images of young, vehicle-, D+Q- group mesenteric artery through an abdominal window exposed to 10 nM Ach. Ach, acetylcholine; BFI, blood flow index. **(J)** the time course of BFI changes. **(K)** summary data of BFI (young n = 6 arteries from 3 mice; old n = 8 arteries from 3 mice; DQ n = 7 arteries from 3 mice). All quantitative data are presented as mean ± SEM. The normality of all quantitative data was verified using the Shapiro-Wilk test. Statistical significance was determined using Kruskal-Wallis followed by Dunn tests for panels D, F, H, and K. The number of biological replicates (n) is indicated in each bar graph. $P < 0.05$ was considered statistically significant.

Furthermore, we also investigated the morphology and size of mitochondria among the groups. We observed that the mitochondria in the aging group displayed characteristics indicative of dysfunction, such as matrix swelling and cristae loss. However, these abnormalities were mitigated in the treatment group ($P < 0.0001$) (Supplementary Fig. 3A-B). Mitochondrial mass is often increased during senescence, potentially resulting from a reduction in mitophagy activity. As dysfunctional mitochondria accrue in senescent cells, the augmented mitochondrial mass may serve as a partial counterbalance for the decline in mitochondrial function.

We confirmed these results by measuring the contribution of D+Q on mitochondria within ECs using a mitochondrial membrane potential and apoptosis detection kit [18]. Our observations revealed that D+Q treatment resulted in the clearance of apoptotic cells and the preservation of mitochondrial membrane potential in ECs derived from aged HUVECs (P14) ($P = 0.0340$) (Fig. 2E-F). We next measured byproducts of mitochondrial metabolism in aged and D + Q-treated ECs. We observed a reduction in levels of mitochondrial ROS and a decrease in fluorescence intensity of dihydroethidium (DHE), a probe for superoxide anion, in both aged HUVECs and mice following D+Q treatment ($P < 0.0001$) (Fig. 2G-H, Supplementary Fig. 3C-D). Furthermore, we employed MitoTracker to label mitochondria and assess mitochondrial quantity, while MitoSOX was utilized to measure ROS levels in HUVECs. By normalizing ROS levels to MitoTracker intensity, we observed that normalized ROS levels were significantly higher in the aged group compared to the D+Q treatment group ($P = 0.0003$) (Supplementary Fig. 3E-F). The GSEA of RNA sequencing data provided insights into the mechanism underlying this phenomenon, suggesting that the clearance of aged cells via apoptosis rather than a reduction in mitochondrial number through mitophagy may explain these observations (Supplementary Fig. 4). In addition, transcriptomic analysis revealed that the expression of canonical mitophagy and mitochondrial biogenesis markers, including PINK1, Parkin, and TFAM, remained unchanged following D+Q treatment compared with aged controls (Supplementary Fig. 4B). Mitochondria-derived ROS can cause damage to cellular components such as proteins, lipids, and DNA, as well as scavenge nitric oxide, leading to eNOS monomerization. Additionally, the monomerization of eNOS to mitochondria can further enhance mito-ROS production due to mitochondrial bioenergetic disturbances [19]. Together, D+Q appears to contribute to the clearance of senescent ECs, which may play a role in the observed effects of the upregulation of eNOS dimerization and the alleviation of mitochondrial ROS in aged mesenteric arteries.

Senolytic treatment improves functional vasodilation responses in aging

Our investigation into the impacts of D+Q therapy on mesenteric artery function revealed its ability to promote the restoration of mitochondrial homeostasis and eNOS dimerization in aged mice. In order to assess the relevance of these findings on intravital vasomotor function, we utilized laser speckle imaging to measure blood flow in the mesenteric arteries via an appendiceal window. In this study, 10 nM acetylcholine was employed to stimulate vasodilation and calculate normalized blood flow. Our results indicate that D+Q-treated mice exhibited significantly greater arterial dilation, resulting in increased blood flow following acetylcholine administration compared to the vehicle group ($P = 0.0221$) (Fig. 2I-K). These findings are consistent with previous studies suggesting that clearance of senescent cells by D+Q may alleviate vasomotor dysfunction in

naturally aging mice [20]. Overall, D+Q therapy potentially contributes to the maintenance of mitochondrial homeostasis and restoration of eNOS dimerization, both of which may influence the observed effects on vascular nitric oxide bioavailability and vasodilation in aged arteries.

DISCUSSION

In conclusion, EC senescence is an important contributor in aging-induced vascular dysfunction. Mitochondrial ROS plays a crucial role in the maintenance of vascular homeostasis, with an excess of ROS leading to decreased nitric oxide bioavailability, thereby inducing cellular nitrosative and oxidative stress. This process can ultimately lead to the dysfunction of eNOS. Additionally, the redistribution of uncoupled eNOS to mitochondria can further enhance ROS production due to mitochondrial bioenergetic disturbances. In this study, our findings collectively reveal that D+Q exerts its protective effects against endothelial senescence and arterial aging through three interconnected pathways. First, D+Q reverses age-related eNOS uncoupling, a key driver of vascular dysfunction, by promoting eNOS dimerization, which directly increases nitric oxide bioavailability and maintains vascular homeostasis. Second, D+Q mitigates mitochondrial dysfunction. Electron microscopy and ROS assays show it restores mitochondrial ultrastructure, reduces excessive mitochondrial mass, and lowers mitochondrial ROS production. This is critical because mitochondrial ROS-induced oxidative stress not only damages cellular components but also exacerbates eNOS monomerization. Third, transcriptomic analysis identifies activation of the relaxin signaling pathway, which is known to mediate vasodilatory, anti-inflammatory, and pro-endothelial survival effects; the upregulation of nitric oxide synthase isoforms in this pathway further reinforces D+Q's ability to enhance nitric oxide signaling. These findings may suggest that the clearance of senescent cells presents a potential therapeutic strategy for improving vascular function in the elderly.

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Conflict of Interest Statement

None declared.

Author contributions

All authors were responsible for interpretation of the data, contributed to the drafting and revised the manuscript critically for important intellectual content. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.0976.

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